CUTTING EDGE

Cutting Edge: An Inducible Sialidase Regulates the Hyaluronic Acid Binding Ability of CD44-Bearing Human Monocytes[1]

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Previous studies established that variable degrees and types of glycosylation can account for differences in the ability of CD44 to function as a receptor for hyaluronic acid. We have now used neuraminidase treatment to conclude that sialylation negatively regulates CD44 on the human monocytic cell line THP-1 and peripheral blood monocytes. Both of these cell types displayed increased receptor activity after overnight culture with LPS. Of particular interest, the sialidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid completely blocked the LPS induced recognition of hyaluronic acid by THP-1 cells. Furthermore, acquisition of this characteristic paralleled induction of one type of sialidase activity. Monocytes may be capable of enzymaticly remodeling cell surface CD44, altering their ability to interact with the extracellular matrix. ***The Journal of Immunology,*** **1999, 162: 5058–5061.**

CD44 represents a family of cell adhesion molecules derived from alternative splicing of a single gene and post-translational modifications of the resulting proteins (1–3). Many potential roles have been proposed for CD44 and related molecules. For example, CD44 has been shown to be involved in hemopoiesis, homing to mucosal lymphatic tissue, and lymphocyte infiltration into inflammatory tissues (4–7). These functions presumably result from the ability of CD44 to interact with extracellular matrix components (3). However, major questions remain about how this activity is regulated.

All isoforms of CD44 have a protein domain that can potentially recognize hyaluronic acid (HA),[3] while particular CD44 variants also interact with fibronectin, collagen, and serglycin (2, 8). Although most blood cells express CD44, few of them use it to recognize HA. Best studied in this regard are lymphocytes, which can be induced to bind HA with activation (9, 10). The HA binding ability of CD44 is likely to be influenced by multiple factors, which include phosphorylation of the cytoplasmic tail, interaction with cytoskeletal proteins, and oligomerization on the cell surface, as well as structural variations in extracellular domains (11–16). It is now clear that glycosylation represents a major means for regulation of CD44 function. While we and others found that *N*- and *O*-linked glycans can prevent the HA recognition ability of CD44, a minimal degree of glycosylation may be required for this activity (15, 17–19). While various CD44 glycoforms can be biosynthetically produced, we now report that the protein may be remodeled through production of a sialidase.

CD44 can be used as a signal transducing receptor, and this is particularly relevant to our study of monocytes. Ligation of monocyte CD44 by Ab or natural ligands triggers production of insulin-like growth factor-1, TNF-α, and IL-1β production (20, 21). Therefore, CD44 could be critical for participation of monocytes in inflammatory responses. It was recently reported that culture of human peripheral blood monocytes (PBMo) with serum or a phorbol ester up-regulated variant CD44 isoform expression and induced HA binding (22, 23). While freshly isolated PBMo do not recognize HA, this is a constitutive property of human alveolar macrophages (22). Exposure to endotoxin and other environmental stimuli in the respiratory tract could modulate this function in monocyte-macrophage lineage cells. We have determined that an inducible sialidase may influence the glycosylation and receptor activity of CD44 on monocytes.

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[3] Abbreviations used in this paper: HA, hyaluronic acid ; FL-HA, fluorescein-conjugated HA ; PBMo, peripheral blood monocyte ; NeuAc2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid ; 4MU-NeuAc, 4-methylumbelliferyl-a-*N*-acetyl-D-neuraminic acid ; SLX, sialosylated Lewis X.

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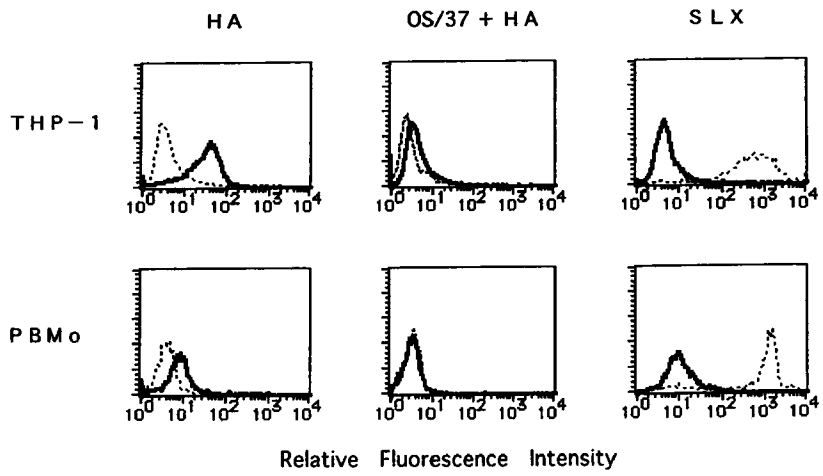

FIGURE 1. The HA binding ability of monocytes is increased by sialidase treatment. THP-1 cells and PBMo were incubated with (bold line) or without (dotted line) sialidase as described in *Materials and Methods*. They were then stained with FL-HA or CSLEX1 (SLX) mAb plus FITC-anti-mouse (IgG+IgM) before analysis with flow cytometry. The results shown in the *left panel* are typical of those obtained in three independent experiments. The *right panel* illustrates the complete loss of staining with an Ab to the SLX Ag as a result of enzyme treatment. CD44-dependent HA binding was confirmed by incubation with the OS/37 mAb before addition of FL-HA (*middle panel*).

Materials and Methods

Cells, cultures, and Abs

THP-1 (24) and PBMC isolated by density centrifugation on Ficoll-Hypaque from normal healthy donors were cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% FCS in the presence (10~10,000 ng/ml) or absence of LPS. The OS/37 mAb to CD44 (pan-CD44) was obtained from Seikagaku (Tokyo, Japan). The CSLEX1 mAb reacts with sialyl derivatives of sialosylated Lewis X (SLX) (25). We found that it provides a sensitive indicator for changes in cell surface glycoforms. Fab FITC-conjugated goat anti-mouse IgM was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Reagents

Sialidase (from *Arthrobacter ureafaciens*), 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (NeuAc2en), and LPS (from *Pseudomonas aeruginosa*) were purchased from Sigma (St. Louis, MO).

Flow cytometry

Sialic acid levels on the cell surface were estimated by staining with anti-SLX Ab reacting to sialyl derivatives. Cells were tested for HA binding by flow cytometry after staining with fluorescein-conjugated HA (FL-HA) (17). As a specificity control, cells were also incubated with the blocking Ab, OS/37, followed by staining with FL-HA. Lymphocytes and monocytes were gated by flow cytometry based on forward and side light scatter. The purity of PBMo were assessed by CD14 staining. More than 95% of PBMC in the monocytes gate and <5% of PBMC in the lymphocytes gate expressed CD14.

THP-1 cells and PBMC were stained with FL-HA and anti-SLX Ab plus PE-conjugated anti-mouse IgM after stimulation with LPS (10 μg/ml) for 24 h and were analyzed by two-color flow cytometry. SLX expression was individually determined on cells gated for the ability to bind or not bind FL-HA. The presence of HA did not influence reactivity of the CSLEX1 mAb with cells (not shown).

Sialidase and sialidase inhibitor treatment

THP-1 and PBMC were treated with bacterial sialidase as previously described (17). They were then stained with FL-HA or anti-SLX mAb plus FITC-anti-mouse (IgG+IgM) before analysis by flow cytometry. As a specificity control, cells were stained with FL-HA after incubation with OS/37. THP-1 and PBMC were cultured with LPS (1 μg/ml) in the presence of increasing concentrations of the sialidase inhibitor, NeuAc2en (4~4000 μM). Percentages of HA binding cells were determined by flow cytometry after staining with FL-HA. OS/37 mAb was used as a specificity control.

Endogenous sialidase assay

Endogenous sialidase activity of LPS-stimulated and untreated THP-1 cells were determined under the optimal conditions described below. The cells (1×10^7) were washed with PBS and sonicated on ice in 9 volumes of ice-cold 0.25 M sucrose containing 1 mM EDTA and 0.2 mM PMSF for 15 s with a mild setting (Sonifier 250; Branson, Danbury, CT). The mixture was centrifuged at 1000 × *g* for 10 min at 4°C, and the supernatant was used as the enzyme fraction. 4-Methylumbelliferyl-α-*N*-acetyl-D-neuraminic acid (4MU-NeuAc; Nakarai, Kyoto, Japan) and bovine-mixed gangliosides (Type II; Sigma) were used as substrates, because 4MU-NeuAc was previously found to be an appropriate substrate for lysosomal-type sialidases and gangliosides for membrane-associated sialidases (26–28). When 4MU-NeuAc was the substrate, the assay mixture contained 40 nmol of the substrate, 10 μmol of sodium acetate buffer, pH 4.6, 200 μg of BSA, and enzyme fractions (20–100 μg protein) in a final volume of 0.2 ml. The incubation was conducted at 37°C for 1–2 h and terminated by the addition of 0.25 M glycine-NaOH, pH 10.4. 4-Methylumbelliferone released was determined fluorometrically as described previously (27). With gangliosides as substrate, the assay mixture was composed of ganglioside substrate (60 nmol as bound sialic acid), 10 μmol of sodium acetate, pH 4.6, 200 μg of BSA, 0.2 mg of Triton X-100, and enzyme in 0.2 ml. After incubation at 37°C for 60 min, the released sialic acid was determined by the thiobarbituric acid method of Aminoff (29). Protein was determined by a dye-binding assay (Bio-Rad, Hercules, CA). One unit of sialidase was defined as the amount of enzyme that catalyzed the release of 1 nmol of sialic acid per hour.

Statistical analysis

The Mann-Whitney test or paired *t* tests were used to determine significant differences.

Results

Glycosylation negatively regulates HA recognition by monocytes

We previously found that sialidase treatment of certain cells or purified CD44-Ig fusion proteins increased their ability to recognize HA (17). Similar experiments were performed with the same enzyme to determine whether terminal sialic acids play a negative regulatory role with respect to CD44 expressed by human monocytes. Exposure of THP-1 cells to sialidase markedly increased their HA binding potential, while viability was unaffected and the recognition was all CD44 mediated (Fig. 1). Effectiveness of the enzyme was also reflected in the complete removal of sialic acid related with SLX. Parallel experiments with freshly isolated monocytes revealed that a subpopulation of cells are capable of HA recognition, but that this potential is masked by terminal sialic acids (Fig. 1).

It has been previously shown that alveolar macrophages, but not peripheral blood monocytes, can bind HA (22). Therefore, HA recognition is a function that might be induced during monocyte activation and/or differentiation. We found that small numbers of normal monocytes and almost half of THP-1 cells could be induced to bind HA by overnight incubation with LPS (see below). Two-color flow cytometry was used to independently analyze SLX Ag expression on cells that did, or did not, have the ability to recognize HA. This was significantly reduced on HA-recognizing THP-1 cells (median fluorescence intensity of HA-binding cells

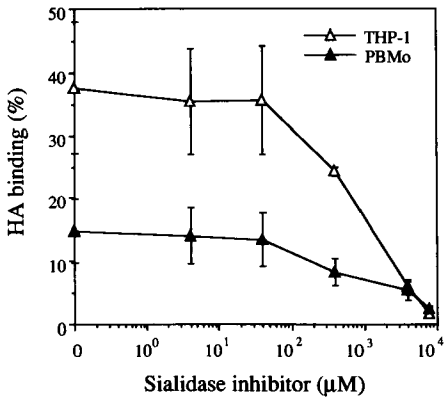

FIGURE 2. Dependence of LPS-stimulated HA recognition by human monocytes on endogenous sialidases. THP-1 cells and PBMC were cultured with LPS (1 μg/ml) in the presence of increasing concentration of the sialidase inhibitor, NeuAc2en (4~8000 μM). Percentages of HA-binding cells were then determined by flow cytometry after staining with FL-HA. The blocking OS/37 mAb was used as a specificity control. The values shown are averaged from three independent experiments.

was 356.4 ± 34.3 as compared with 479.1 ± 69.7 for the HA-nonbinding cells; $p < 0.05$) or PBMo (median fluorescence intensity of 610.7 ± 54.3 for HA-binding cells vs 893.9 ± 52.1 for HA-nonbinding monocytes; $p = 0.001$). Thus, decreased display of a sialylated surface protein correlated with acquisition of HA recognizing ability.

A sialidase inhibitor diminishes the LPS-induced HA binding ability of monocytes

It has recently been suggested that endogenous sialidase activity may contribute to the ability of activated B cells to effectively interact with T lymphocytes (30). We thought it possible that inducible sialidases also regulate CD44-mediated functions of monocytes. Accordingly, NeuAc2en was included in cultures of LPS-stimulated THP-1 cells and normal PBMo. While the drug inhibited LPS induction of HA recognition on both cells in a dose-dependent manner, there was no effect on cell viability (Fig. 2 and data not shown). The small downward shifts in SLX Ag expression that were induced by LPS were blocked by the sialidase inhibitor (not shown). These findings indicate that endogenous sialidase activity may contribute to inducible HA recognition in human monocytes.

LPS elevates sialidase activity in THP-1 cells

Mammalian cells are known to possess several types of sialidase (26–28), and we sought direct evidence for inducible activity in monocytes. Two substrates that distinguish sialidase types were used to measure activity in LPS-induced THP-1 cells. There was significantly (8 h; $p = 0.0058$, 16 h; $p = 0.0032$) induced activity toward 4MU-NeuAc, reflecting the presence of lysosomal-type sialidases (Fig. 3*A*). Parallel analyses revealed good kinetic concordance between the appearance of this activity and increased ability of THP-1 cells to bind HA (Fig. 3*B*). In contrast, we recorded no increment in sialidases capable of cleaving gangliosides in the presence of Triton X-100 (Fig. 3*A*). This assay condition detects plasma membrane-type sialidase (26–28). We conclude that LPS induces expression of a lysosomal type of sialidase activity in this monocyte cell line. The kinetics of appearance are consistent with a relationship between endogenous sialidase activity and CD44-mediated HA recognition.

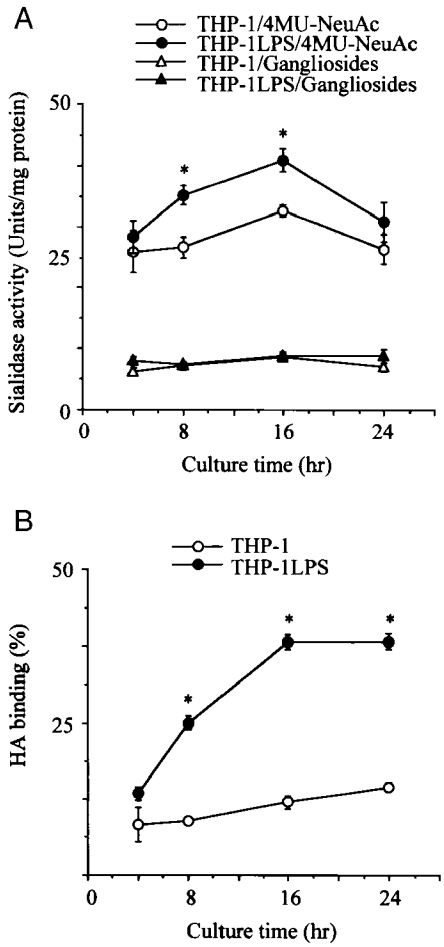

FIGURE 3. LPS induces lysosomal-type sialidase activity in monocytes. THP-1 cells were cultured with or without LPS (10 μg/ml) for 4–24 h. Cells were harvested and enzyme activity was determined using 4MU-NeuAc or gangliosides as described in *Materials and Methods* (*A*). In parallel, percentages of HA-binding cells were measured by flow cytometry (*B*). Preincubation of OS/37 mAb was used to determine background staining. Values shown are averaged from 10 independent experiments. The asterisks indicate significant differences ($p < 0.01$).

Discussion

Glycosylation of CD44 represents one of several means through which cells control their ability to interact with HA, and this can change in response to activating stimuli (17, 18, 31). While mechanisms associated with such changes are poorly understood, our data suggests that an inducible sialidase may be involved.

We now confirm and extend previous studies demonstrating that cytokines and other agents can induce HA recognition by monocytes (23, 32). Freshly isolated monocytes differed in the degree to which HA binding could be induced by LPS as compared with an established monocyte cell line. This is similar to previous demonstrations that only a subset of activated B cells are induced to recognize HA (9, 17) and presumably reflects some type of heterogeneity in monocytes in peripheral blood. We note that LPS induction leads to smaller degrees of HA recognition and loss of the SLX Ag than does enzymatic removal of terminal sialic acids. Therefore, endogenous sialidase or other mechanisms may only partially remodel/replace the cell surface CD44 on activated monocytes. Several studies revealed that ligation of CD44 molecules on monocytes leads to inflammatory cytokine release (20,

21). Therefore, acquisition by monocytes of the ability to recognize HA in tissues is likely to have important consequences and the molecular basis for this transition merits study.

Endogenous sialidase activity controlled by the Neu-1 locus in mice was suggested to play a regulatory role in the recognition of Ia molecules in T cells by removal of sialic acids from some surface molecules (33, 34). A human lysosomal sialidase gene was recently cloned and mapped to the same segment of DNA as the murine Neu-1 locus (35–37). This type of enzyme is thought to be important for removal of terminal sialic acids from glycoproteins in cooperation with lysosomal proteases (26). Many cell surface receptors are known to be capable of either constitutive or ligand-induced endocytosis and recycling back to the cell surface (38, 39). Our findings raise the possibility that sialidase contributes to the intracellular remodeling of CD44. While lysosomal enzymes could also be secreted, we were unable to detect sialidase activity in supernates of LPS-stimulated THP-1 cells (data not shown). Lysosomal sialidase could play an essential role in the removal of terminal sialic acid residues from cell surface glycoproteins and may be important in regulation of the immune system. Further study should reveal additional details about the fate of monocyte surface proteins and whether this new mechanism is used by other cell types.

It is important to stress that multiple molecular mechanisms are used to control CD44-mediated functions. Each domain of the protein contributes to structural integrity and all domains are subject to cell-type specific variation (13). Although the ability to recognize HA can be influenced in a positive or negative way by *N*-glycan addition, *O*-linked glycosylation can also be important (15, 17–19, 40). Oligomerization of the protein on the cell surface via intrachain disulfide bonds or interactions with other proteins can also be an important determinant of receptor activity (12, 14, 16, 41). Therefore, differential utilization of sialidases is unlikely to account for all cases where cells differ in ability to recognize HA. However, sialidases appear to influence monocyte functions, and the experimental approaches we describe should be valuable for the assessment of other cell types.

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